REMARKS

Claims 1-74 have been canceled, and 75-146 are pending for the Examiner's consideration. Claims 75, 80, 81, 85, 86, 89, 90, and 93-95 are amended herein. New claims 96-146 have been added. Applicant respectfully submits that these amendments introduce no new matter. Support for the new claims can be found in the specification and original claims. Applicant respectfully requests entry of the present amendment in view of the Request for Continued Examination ("RCE") filed herewith.

Applicants' attorneys would like to thank Examiner Dentz for kindly granting an interview on September 29, 2005.

I. Claim Amendments and Support

Applicants submit that the amended and new claims are supported in the specification and claims and support for selected language is as follows: Claims 75 and 85 to an isolated mixture of theaflavin is supported by Example 1 on pages 19-20, the bridging paragraph on pages 4-5; and the bridging paragraph on pages 9-10, Claims containing the term "pharmaceutically acceptable vehicle" and types of vehicles is supported on page 6, 1st complete paragraph; Claims containing forms of pharmaceutical compositions, such as capsules, stabilizers, agents, oils are supported beginning on page 15, under Pharmaceutical Compositions; Claims containing range of %s for the 4 theaflavins is supported on page 10 , 2nd complete paragraph and on page 15, last paragraph; and Claims directed to soft gels with oil are supported by Example 3.

II. Rejection Under 35 U.S.C. § 102(b)

Claims 75-95 were rejected under 35 U.S.C. § 102(b) as anticipated by Vitasyn, DE 19627344 A1 (translation) for the same reasons that previous claims 15, 16, 28, 29, 34, 35, 38, 58 and 59 were anticipated in the previous office action. The Examiner alleges that Vitasyn

discloses the benefits of the polyphenol constituents of both green tea and its fermented form, which is black tea, for the treatment of hyperlipidemia. The Examiner further cites page 2 of the translation and claims 1, 14 and 18 to support his rejection. Applicants respectfully traverse this rejection and wish to point out that the claims are directed to a method of reducing LDLs while not significantly reducing HDLs comprising administering **an isolated mixture** of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin 3,3'-digallate or **a composition consisting essentially of a mixture** of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin 3,3'-digallate or another recited amount or formulation of the 4 recited theaflavins in a therapeutically effective amount and for a time period sufficient to reduce the LDL while not significantly reducing the HDL over the time of administration, and a daily dosage composition for oral administration, which comprises such mixtures and compositions of the 4 recited theaflavins. in a therapeutically effective amount and for a time period sufficient to reduce the LDL while not significantly reducing the HDL over the time of administration.

The Examiner refers to claim 1 of Vitasyn as disclosing a pharmaceutical composition containing theaflavins and claims 14 and 18 for its use in treating hypercholesteremia and lipid metabolism, respectively. However, all that Vitasyn's preparation discloses is a laundry list of substances. Vitasyn does not disclose a method of treating hyperlipidemia with a composition that contains specific mixtures of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin 3,3'-digallate resulting in the recited outcome that reduces LDLs while not significantly reducing HDLs. Applicants have added additional independent and dependent claims to encompass theaflavin mixtures useful in the present invention. These additional claims cover compositions containing the theaflavins at specific percentage ranges or dosage amounts.

Applicants submit that the amended claims that are presently pending are not anticipated by Vitasyn and it is requested that this rejection be withdrawn.

III. Rejection Under 35 U.S.C. § 103

Claims 75-95 were rejected under 35 U.S.C. § 103 as obvious over Vitasyn, DE 19627344 A1 (translation) for the same reasons that previous claims 15, 16, 28, 29, 34, 35, 38, 58 and 59 were considered to be obvious in the previous office action. The Examiner alleges that it would be obvious to treat hypercholestermia and the other enumerated diseases with the enumerated theaflavin ingredients. For the same reasons that the pending claims were not anticipated by Vitasyn's preparation as argued above, it is not obvious to treat a human to reduce the LDLs but have no significant reduction in the HDLs using the claimed mixtures of theaflavins. The claims disclose specific theaflavin compositions that are not suggested by Vitasyn's preparation nor does Vitasyn suggest such treatment could be expected to result in reduction in LDLs and no significant reduction in HDLs as claimed by applications. For the foregoing reasons, it is requested that this rejection be withdrawn as to the pending claims.

IV. Rejection Under 35 U.S.C. § 102(b)

Claims 75-95 were rejected under 35 U.S.C. § 102(b) as anticipated by Abe *et al.*("Abe") for the same reasons that previous claims 15, 16, 28, 29, 34, 35, 38, 58 and 59 were anticipated in the previous office action. The Examiner alleges that Abe discloses that theaflavins in black tea are excellent inhibitors of squalene epoxidase, the rate-limiting enzyme of cholesterol biosynthesis, and concludes that the method of treating hyperlipidemia by administering to a patient a theaflavin mixture is anticipated. Applicants respectfully traverse this rejection and would like to point out that Abe discloses an *in vitro* assay to show that in the presence of

theaflavin, squalene monooxygenase activity is inhibited, the inhibition concentration is 1 μM to 5 μM (600 μg to 3,000 μg)/liter. Abe simply does not disclose applicants' claimed method of treatment or applicants' dosage form. Therefore, Able does not anticipate the presently pending claims to a method of reducing LDL in a human subject and a daily dosage form to treat the subject. It is requested that this rejection be withdrawn.

V. Rejection Under 35 U.S.C. § 103)

Claims 75-95 were rejected under 35 U.S.C. § 103 as obvious over Abe *et al.*("Abe") for the same reasons that previous claims 15, 16, 28, 29, 34, 35, 38, 58 and 59 were considered to be obvious in the previous office action. The Examiner alleges that Abe discloses that black tea would be expected to be an excellent beverage to lower cholesterol, and thus using theaflavins as presently claimed would be obvious. Again Abe does not disclose treating humans or disclose the claimed daily dosage for treatment. As squalene monooxygenase is one of many enzymes involved in cholesterol biosynthesis, it is our position that Abe's results are not predictive of the effect of theaflavin on the inhibition of cholesterol biogenesis *in vivo* with the same mechanism, and there has been continuing research to support this unpredictability *in vivo*.

First of all, experiments conducted *in vivo* determined that theaflavins do not go into the body as theaflavins. Theaflavins go through biotransformation in the digestion system (See Exhibit A, Jhoo *et al.*, 2005). This study shows that when theaflavin goes through the digestion system, it becomes a molecule called theanaphthoquinone. The Jhoo study shows that very low levels of theaflavins can be detected in the serum. Therefore, it is unlikely that the squalene monooxygenase that is found in liver cells will not come in contact with theaflavins molecules. Therefore, the Jhoo study suggests that Abe's results will be difficult to repeat *in vivo*, and thus

renders the outcome unpredictable. In further support of this position, another study shows that after administration of theaflavin, only minute amounts of theaflavin can be detected in serum (See Exhibit B - Mulder *et al,* 2001, page 271, the last sentence in the abstract; page 278, first paragraph –). Again with such minute levels of theaflavin, it is not likely that theaflavin in the body would come in contact with squalene monooxygenase to inhibit this enzyme and cause a reduction in cholesterol synthesis *in vivo*.

An additional point supporting unpredictability is that it is known that theaflavin is very unstable and degrades into other forms in the digestion system (See Exhibit C - Su *et al,* 2003, page 192, where the pH in intestine is basic and theaflavin will be degraded into metabolites very quickly).

Applicants submit that one cannot use the results from *in vitro* studies to simply predict the results *in vivo*, particularly with the Abe study which analyzed the inhibition of squalene monooxyygenase in a test tube. Without more evidence of use in an animal or human or higher *in vivo* levels or stability, applicants submit that the results in Abe are not predictive of the outcome in human subjects.

The present invention discloses the unique lipid profile of animals and humans as the result of the administration of theaflavins. Applicants have shown that the oral administration of theaflavins results in a different effect on each lipid component. Applicants' results demonstrate that there is no change of HDL cholesterol and triglycerol levels. However, total cholesterol level is lowered about 11%, and LDL level is lowered by 17%. LDL is the risk factor that is most closely linked to cardiovascular diseases. So the finding has clinical significance.

In summary, the effect of theaflavin on the cholesterol panel is very complicated. Research supported by Exhibits A-C, and submitted herewith, has shown that theaflavin has very

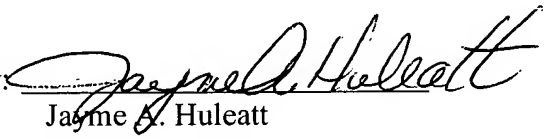
low bioavailability in animals, is present in minute quantities in serum and has a very low likelihood of coming into contact with squalene monooxygenase *in vivo*. Therefore, Abe does not provide a motivation or suggest to a person skilled in the art that theaflavin would be useful for lowering LDL while not significantly reducing HDL. In view of these arguments, it is requested that this rejection be withdrawn to the presently pending claims.

Conclusion

All of the stated grounds of rejection have been properly traversed or rendered moot. Applicants therefore respectfully request that the Examiner reconsider and withdraw all presently outstanding rejections. Applicants believe that a full and complete response has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment is respectfully requested.

Respectfully submitted,
COOLEY GODWARD LLP

By: 
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Stability of Black Tea Polyphenol, Theaflavin, and Identification of Theanaphthoquinone as Its Major Radical Reaction Product

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In the current study, we have focused on isolation and detection of major radical oxidation products from theaflavin in order to better understand antioxidation mechanisms of this compound. Theanaphthoquinone was identified as a major oxidation product of theaflavin from two different oxidant model systems: DPPH and peroxidase/hydrogen peroxide. This result indicated that the benzotropolone moiety in theaflavin may play an important role in its antioxidant properties. The stability of theaflavin was studied in varying pH solutions: simulated gastric juice and buffer solutions of pH 5.5, pH 7.4, and pH 8.5. The results indicated that theaflavin is unstable in alkaline conditions, while it was stable in acidic conditions. Theanaphthoquinone was identified as an autoxidation product of theaflavin during its stability study in alkaline conditions.

KEYWORDS: Theaflavin; theanaphthoquinone; oxidation; radical oxidation; pH stability

INTRODUCTION

As a popular beverage, tea (*Camellia sinensis*) has attracted public attention because of accumulating scientific evidence linking tea consumption with health benefits (*1*). Fresh tea leaves contain four major tea catechins with unique biological activities; however, black tea is manufactured through fermentation of the tea leaves. During the fermentation process, important chemical changes occur due to the action of polyphenol oxidase (PPO). PPO is responsible for oxidizing the dihydroxylated B ring (catechol) and trihydroxylated B ring (pyrogallol) of tea catechins to their *o*-quinones. Subsequent chemical reactions generate the various characteristic black tea polyphenolic pigments that are not found in unprocessed tea leaves. Four major theaflavins have been identified from black tea, namely, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (*2*).

Tea consumption may provide health benefits, and these biological activities are believed to arise from the antioxidant activity of polyphenolic compounds in tea, specifically their ability to effectively scavenge reactive oxygen species (*1*). A number of studies have demonstrated the antioxidant capacity of black tea and black tea polyphenols using in vitro and in vivo methods. Many in vitro studies have shown that black tea has a comparable antioxidant capacity to green tea. Researchers using the oxygen-radical absorbance capacity assay have shown that black tea has a similar peroxyl radical scavenging capacity to green tea (*3*). Trolox equivalent antioxidant capacity values of green tea and black tea are comparable (*4*). Black tea extracts have been shown to exhibit protection of human red blood cells against oxidative damage from lipid peroxidation (*5*). Sarkar and Bhaduri (*6*) reported that black tea extracts are more effective than green tea catechins in scavenging superoxide anions.

Several studies have reported a proposed mechanism for the reaction of tea catechins with radicals and have proposed mechanisms for the antioxidant properties of these compounds (*7*, *8*). When tea catechins were reacted with peroxyl radical generated by thermolysis of the radical initiator 2,2′-azobis-(2,4-dimethylvaleronitrile), the resulting seven-membered B ring anhydride and symmetrical dimer were identified as the oxidation products of EGCG and (−)-epigallocatechin (EGC) (*8*). These results indicated that the principal sites for radical reaction are on the B ring rather than the galloyl moiety. Although different oxidation model systems, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), gave different oxidation products, Sang et al. have demonstrated that the catechin B ring is the preferred site for oxidation (*9*). Theaflavins have been regarded as biologically important active components in black tea. Little information is available concerning a mechanism for the antioxidative action of black tea polyphenols. Because the antioxidant mechanisms of theaflavins are still unclear, we sought to identify the oxidation products from theaflavin in order to better understand these mechanisms. Identification of the oxidation products of theaflavins can provide useful information to understand the mechanism giving rise to the antioxidant

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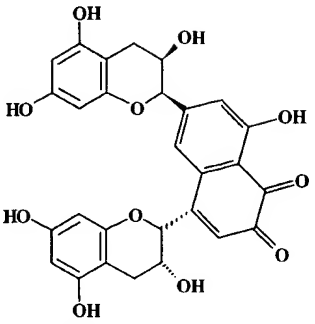

Figure 1. Chemical structure of compound 1.

properties of these molecules. Also, limited information is available about the stability of theaflavins in various pH solutions. In this study, we focused on examination of the stability of theaflavin in different pH conditions.

MATERIALS AND METHODS

Materials and General Procedures. Epicatechin (EC), DPPH, and horseradish peroxidase were purchased from Sigma (St. Louis, MO). All high-performance liquid chromatography (HPLC) grade solvents were from Fisher Scientific (Fair Lawn, NJ). Tea catechin, EGC, was isolated from commercial green tea polyphenol extract using an LH-20 column chromatography. Thin-layer chromatography (TLC) was performed on Sigma-Aldrich silica gel TLC plates (250 μm thickness, 2−25 μm particle size), and the spots were detected by UV illumination and spraying with 5% (v/v) H_2SO_4 in an ethanol solution. ^{1}H NMR spectra were obtained on a Varian 600 instrument (Varian Inc., Palo Alto, CA). The compound was analyzed in acetone-d_6 with tetramethylsilane as an internal standard. Atmospheric pressure chemical ionization (APCI)-MS were recorded on a Micromass Platform II system (Micromass Co., Beverly, MA) equipped with a Digital DECPC XL 560 computer for data analysis.

Theaflavin Preparation. Theaflavin was prepared by a modified enzymatic oxidation method from Tanaka et al. (*10*). In brief, crude PPO was prepared from commercial banana fruit. Fresh banana flesh (400 g) was homogenized with 800 mL of cold 100 mM potassium phosphate buffer (pH 7.0, 4 °C). The homogeneous solution was centrifuged at 4 °C for 20 min (10000*g*), and clear supernatant was collected. The same volume of cold acetone (−40 °C) was slowly poured into the collected solution with stirring. Then, precipitated proteins were collected by centrifugation (10000*g*, 20 min, 4 °C). The resulting pellets were washed with the same buffer three times and dissolved in the same buffer. The protein solution was lyophilized; the resulting powder was used as a crude PPO.

EC (1 g) and EGC (1 g) were dissolved in 200 mL of phosphate−citrate buffer (50 mM, pH 5.0) along with 2 g of crude PPO enzyme. The enzymatic oxidation was carried out at room temperature for 6 h with stirring. The reaction solution was then subjected to partition three times with the same volume of ethyl acetate. The organic layer was concentrated under reduced pressure. The resulting residues were subjected to Sephadex LH-20 and RP-18 column chromatography successively to afford a reddish theaflavin (yield, 15.2%).

Theaflavin Oxidation Model Using DPPH Oxidant System. Theaflavin (720 mg) was dissolved in 30 mL of acetone, and the radical oxidation was initiated by adding DPPH (1000 mg) into this solution. The reaction was carried out for 20 h with stirring in the dark at room temperature. The reaction solution was dried under reduced pressure. The resulting residue was subjected directly to Sephadex LH-20 column chromatography eluting with 95% ethanol. Six fractions were collected (each 200 mL), and fraction 3 was subjected to LH-20 (95% ethanol) and RP-18 (50% aqueous methanol) successively for further purification and yielded a deep reddish amorphous solid, theanaphthoquinone (24 mg). Further purification of fraction 5 afforded unreacted theaflavin (155 mg).

Theaflavin Oxidation Model Using Peroxidase/Hydrogen Peroxide Oxidant System. Theaflavin (500 mg) was dissolved in 30 mL of 20% aqueous acetone along with 1 mg of peroxidase. One milliliter of 3.11% hydrogen peroxide solution was added to the reaction solution every 40 min. The reaction was carried out at room temperature for 3.5 h with stirring. Then, the reaction solution was dried under reduced pressure. The resulting residues were subjected to Sephadex LH-20 column chromatography eluting with 95% ethanol. Among the collected seven fractions (each 180 mL), fraction 3 was subjected to LH-20 (95%

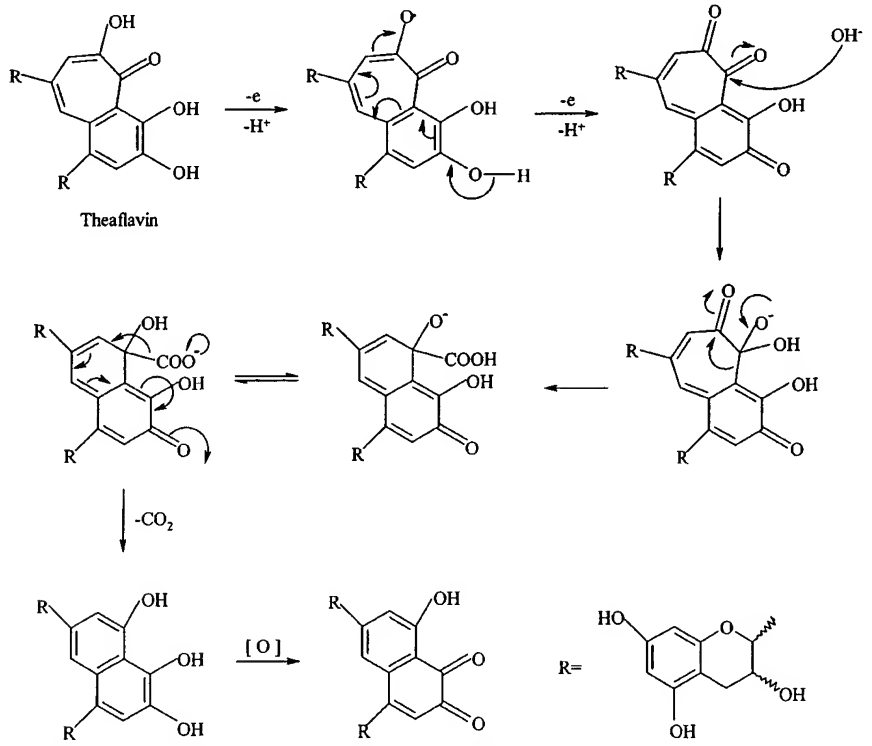

Figure 2. Proposed mechanism of theanaphthoquinone formation in radical reaction.

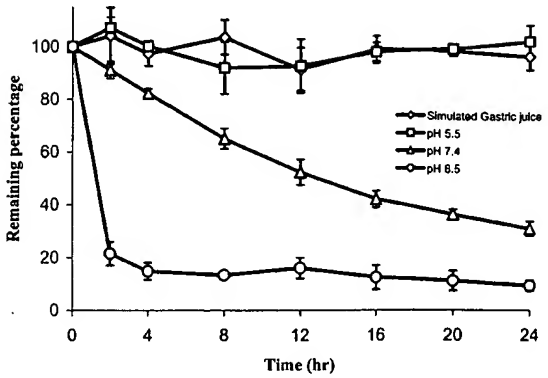

Figure 3. Theaflavin degradation in different pH solutions. Data were expressed as means ± SD ($n = 3$).

ethanol) and RP-18 (50% aqueous methanol) column chromatography to afford theanaphthoquinone (35 mg). Fractions 5 and 6 were combined and subjected to RP-18 column chromatography eluting with a gradient of 40−50% of aqueous methanol to yield unreacted theaflavin (123 mg).

Theanaphthoquinone. Deep red amorphous powder. APCI-MS [M + H]$^+$ at *m/z* 535. ^{1}H NMR (600 MHz in acetone-d_6): δ_H 7.40 (1H, s), 7.28 (1H, s), 6.74 (1H, s), 6.01 (1H, d, $J = 2.4$ Hz), 6.01 (1H, d, $J = 2.4$ Hz), 5.96 (1H, d, $J = 2.4$ Hz), 5.96 (1H, d, $J = 2.4$ Hz), 5.35 (1H, br s), 5.11 (1H, br s), 4.40 (1H, d, $J = 3.6$), 4.37 (1H, br s), 2.68−2.93 (4H, m).

Theaflavin Stability in Various pH Solutions. The stability of theaflavin (4 mg in 16 mL) was examined in different pH solutions, such as simulated gastric juice (0.2% sodium chloride, 0.24% hydrochloric acid), pH 5.5 sodium acetate buffer (60 mM), pH 7.4 phosphate buffer (60 mM), and pH 8.5 phosphate buffer (60 mM). Aliquots (1 mL) of the sample were collected and analyzed at various time intervals using HPLC to examine the degradation rate of theaflavin at ambient temperature. The HPLC system was fitted with a Zorbax ODS HPLC column (5 μm, 4.6 mm × 250 mm, RP-18) and equipped with an autosampler (Waters, 717) and UV detector (Varian, 2050) at a wavelength of 375 nm. Theaflavin analysis was performed with a linear increasing gradient from 10 to 60% acetonitrile in water with constant 0.1% acetic acid in 15 min. The flow rate was 1.0 mL/min. The degradation rate of theaflavin was measured based on decreasing theaflavin peak area.

To identify oxidation products of theaflavin in alkaline conditions, an aliquot of sample in pH 8.5 was partitioned with ethyl acetate, and the organic part was dried under reduced pressure. The sample was dissolved in water and analyzed with LC/electrospray ionization (ESI). The analysis was performed on the Finnigan TSG 7000 mass spectrometer equipped with an HP 1100 HPLC system (Hewlett-Packard, Palo Alto, CA). The mass spectrometer was operated in the negative ESI mode. Full scans were acquired from *m/z* 100 to 650/s. The information for MS condition is as follows: voltage, 3.5 kV; capillary temperature, 300 °C; sweep gas, 70 psi; and auxillary gas, 5 units. HPLC was performed with a Prodigy ODS(3) HPLC column (5 μm, 100 Å, 2.0 mm × 250 mm, Phenomenex, Torrance, CA). The mobile phase delivered at 0.2 mL/min was a linear gradient from 5 to 95% acetonitrile in water with constant 0.1% formic acid in 40 min.

RESULTS AND DISCUSSION

Identification of Theaflavin Oxidation Product from DPPH and Peroxidase/Hydrogen Peroxide Oxidant Model Systems. A major oxidation product of theaflavin was isolated using column chromatography methods from two different oxidant model systems, DPPH and peroxidase/hydrogen peroxide, and its structure was identified by interpretation of ^{1}H NMR and MS spectra. It has been reported that the dihydroxy B ring and the trihydroxy B ring are the major sites for antioxidant action of tea catechins (*7*). As theaflavin is dimeric compound formed from the catechin B ring, it was necessary to identify the principal site of oxidation that gives rise to the antioxidant properties of these compounds. Therefore, we carried out a theaflavin oxidation experiment using two different oxidation model systems. The DPPH is a stable radical, which is widely used for determination of the antioxidant activity of test compounds. The DPPH radical forms a reduced DPPH paired with a hydrogen from the test compounds. Heme-containing peroxidases reduce hydrogen peroxide to water, while oxidizing various substrates. The horseradish peroxidase/hydrogen peroxide system has been used to generate substrate-derived radicals that can undergo further reactions.

Theaflavin was subjected to oxidation using the above two model systems, and successive purification steps using LH-20 and RP-18 column chromatography afforded a compound (**1**) from both oxidation systems. After comparison with the ^{1}H NMR spectrum of theaflavin and those of isolated compound **1** along with published spectral data (*11*), it was found that the chemical shift of the characteristic three proton signals in the benzotropolone moiety of compound **1** was different from those of theaflavin. Specifically, the chemical shifts of three aromatic protons in the benzotropolone moiety of theaflavin were δ_H 8.04 (1H, s), 8.01 (1H, s), and 7.58 (1H, s); however, those of compound **1** were δ_H 7.40 (1H, s), 7.28 (1H, s), and 6.74 (1H, s). When we compared the ^{1}H NMR spectrum of compound **1** with those of theanaphthoquinone, which was reported by Tanaka et al. (*12*), we concluded that the spectral characters of this compound were consistent with those of theanaphthoquinone (**Figure 1**). Moreover, positive ion APCI-MS ([M + H]$^+$ at *m/z* 535) data of compound **1** strongly supported this conclusion. This compound has been reported as an oxidation product of theaflavin by Tanaka et al. (*10*). They reported that this compound is both an enzymatically and a nonenzymatically generated from theaflavin. As shown in **Figure 2**, we propose a radical oxidation mechanism of theaflavin and theanaphthoquinone formation through one-electron oxidation. From these results, it is probable that the benzotropolone moiety of theaflavin plays an important role in affording antioxidant protection for the preferred oxidation site. Jovanovic et al. (*13*) reported that although theaflavin radicals have a higher reduction potential than the tea catechin EGCG, theaflavins have significantly higher reaction rates with superoxide radicals than EGCG. These authors proposed that the benzotropolone moiety might be responsible for electron donation because of the existence of resonance forms. As part of our continuing chemical study of the antioxidant mechanisms of theaflavins in our laboratory, A ring fission products of theaflavin 3,3′-digallate have been reported using the hydrogen peroxide oxidant system (*14*). This result indicated that the preferred oxidation site of theaflavin 3,3′-digallate is placed in the A ring of flavan-3-ol rather than the benzotropolone moiety. Although the experiment was carried out with a different oxidant system, this observation suggests that gallated theaflavins may have a different antioxidation mechanism as compared to nongallated theaflavins. Interestingly, Jovanovic et al. (*13*) reported that gallated theaflavins have a lower superoxide scavenging activity than nongallated theaflavin. They proposed that the gallate moiety may prevent reaction between the radicals and the benzotropolone moiety in theaflavins. It might be that further chemical studies are needed to understand the antioxidation mechanism of monogallated theaflavin.

Stability of Theaflavin in Different pH and Its Oxidation Product. In the present study, solutions with various pH values, including simulated gastric juice and buffer solutions of pH 5.5,

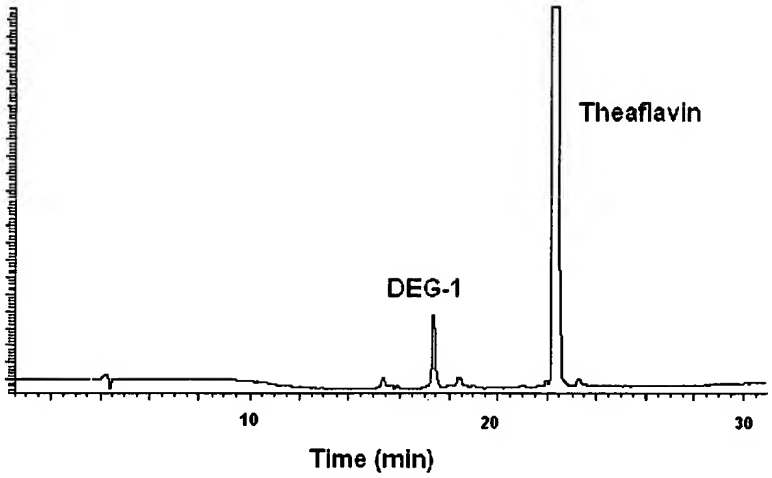

Figure 4. Theaflavin degradation product at alkaline condition.

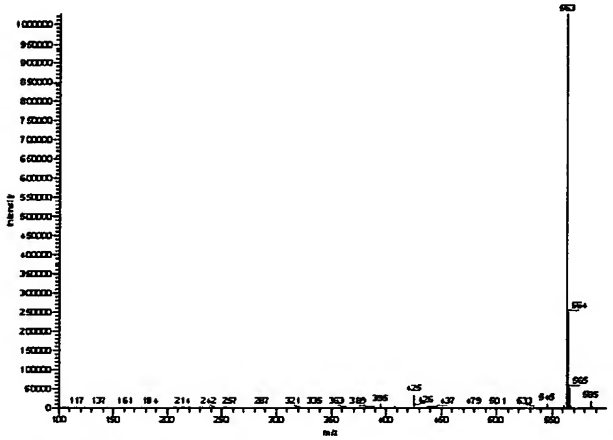

Figure 5. ESI spectrum of theaflavin.

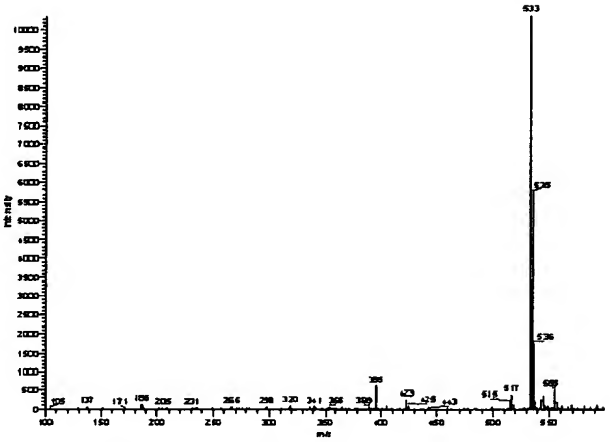

Figure 6. ESI spectrum of DEG-1.

pH 7.4, and pH 8.5, were chosen to examine the stability of theaflavin in acidic and alkaline conditions. It has been reported that plant flavonoids are vulnerable in alkaline condition, and several studies have demonstrated that tea catechins are unstable in neutral and alkaline pH (*15*−*17*). For example, the tea catechins, EGC and EGCG, are completely degraded in pH 7.4 phosphate buffer solution within 3 h (*17*). Interestingly, EGC and EGCG, which contain pyrogallol in the B ring, were much less stable than tea catechins EC and ECG, which have a catechol B ring. One reason for the difference is that pyrogallol-containing catechins form semiquinones more readily than those containing the catechol moiety (*17*).

When theaflavin was incubated in the acidic solutions, pH 5.5 buffer solution, and simulated gastric juice, as shown in **Figure 3**, this compound exhibited a high degree of stability during a 24 h incubation period. In contrast, theaflavin was unstable in alkaline conditions. At pH 7.4 buffer solution, 34.8% of theaflavin was degraded after 8 h of incubation. Moreover, when theaflavin was added into pH 8.5 buffer solution, it rapidly changed to a dark brown color, and 78.4% of theaflavin was degraded after 2 h of incubation.

During the course of this stability study, it was noteworthy that a major degradation product of theaflavin was identified during HPLC analysis in alkaline conditions: pH 7.4 and pH 8.5 (**Figure 4**). To identify the degradation product, the solution was partitioned with ethyl acetate and the organic part was analyzed with LC/ESI. **Figure 5** represents the negative ion ESI spectrum of theaflavin (*m/z* 563 $[M - H]^-$). The LC/ESI experiment revealed that the DEG-1 has a molecular ion at *m/z* 533 $[M - H]^-$ (**Figure 6**). In **Figure 6**, the pseudo-molecular ion peak at *m/z* 535 $[M - H]^-$ was also identified as a result of the reduction product. This result indicated that the spectral character of DEG-1 was the same as theanaphthoquinone reported by Tanaka et al. (*12*). After comparison of HPLC analysis of an isolated theanaphthoquinone standard and DEG-1, we concluded that DEG-1 is theanaphthoquinone. This observation might be important to the black tea beverage industry. It is possible that theaflavin in black tea beverage undergoes a change to theanaphthoquinone in aqueous solution. To date, little scientific information is available about the biological activities of theanaphthoquinone. It might be interesting to understand its biological activity and to examine its presence in commercial black tea beverage products.

In conclusion, theanaphthoquinone was identified as an oxidation product of theaflavin using DPPH and peroxidase/hydrogen peroxide oxidant model systems. This indicated that the benzotropolone moiety in theaflavin may play an important role in its antioxidant activity. The stability of theaflavin was examined in solutions of varying pH. The results confirmed that theaflavin is unstable in alkaline conditions in agreement with a recent report of Su et al. (*18*). In contrast, theaflavin was stable in acidic condition. Theanaphthoquinone was identified as an autoxidation product of theaflavin during its stability study in alkaline conditions. As shown in **Figure 2**, the formation of theanaphthoquinone from theaflavin can be facilitated in alkaline conditions.

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Analysis of theaflavins in biological fluids using liquid chromatography–electrospray mass spectrometry

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Abstract

A HPLC–MS procedure for the sensitive and specific analysis of the black tea flavonoid theaflavin in human plasma and urine was developed. Levels were measured after enzymatic deconjugation, extraction into ethyl acetate, and separation by HPLC, using tandem mass spectrometry as a detecting system. Two healthy volunteers consumed 700 mg theaflavins, equivalent to about 30 cups of black tea. The maximum concentration detected in blood plasma was 1.0 $\mu g\ l^{-1}$ in a sample collected after 2 h. The concentration in urine also peaked after 2 h at 4.2 $\mu g\ l^{-1}$. Hence, only minute amounts of theaflavins can be detected in plasma and urine samples of healthy volunteers after ingestion. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Theaflavins

1. Introduction

Flavonoids are widely distributed in the plant kingdom and over 4000 different flavonoids have been identified [1]. Most of these compounds are strong anti-oxidants that can be found in vegetables, fruits, wine and tea. Epidemiological studies in humans indicate that a high intake of flavonoids is associated with a reduced risk of coronary hart disease [2] and stroke [3]. Although tea consists for over 99% water, it is also a very rich source of flavonoids. There is a large interest in the effect of tea flavonoids on health [2,3], but remarkably little is known about the bioavailability. The main flavonoids found in green tea are (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate. Studies with ^{14}C-labelled (+)-catechin showed that this compound is absorbed from the intestine [4]. Once absorbed, biotransformation enzymes in the gut wall and the liver extensively modify catechins [4–6]. Recently, methods for the measurement of unlabelled, native (−)-epicatechins, in blood plasma and urine were developed and the absorption of catechins in rats and humans was confirmed [7,8].

During the fermentation step in the production of black tea, most of the catechins are oxidised and condensed into theaflavins (dimers) and thearubigins (polymers?) [9]. Of the flavonoids in black tea, approximately 30% are catechins, 10% are theafl-

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avins, and 50% are thearubigins [9]. In most western countries, people drink black tea and consequently black tea accounts for approximately 80% of the world tea consumption. The bioavailability of the typical black tea flavonoids, theaflavins and thearubigins, however, has not been studied.

The identification and quantification of low levels (μmol l^{-1}–nmol l^{-1}) of flavonoids in complex matrices such as biological fluids is very difficult. Elegant analytical methods for the detection of catechins [7] and quercetin [10] in plasma and urine were developed, but these methods are more or less specific for the flavonoid studied. Because more than 4000 different flavonoids are known [1], a more generic approach for the analysis of these compounds is needed. Recently Andlauer et al. [11] showed that 19 different flavonoids, and closely related compounds, can be analysed in plant extracts using high-performance liquid chromatography (HPLC) combined with electrospray ionisation mass spectrometry (ESI-MS). The combined separation power of liquid chromatography (LC) and MS makes this type of technique a more generally applicable tool for screening all kinds of biological matrices for flavonoid content. Sägesser and Deinzer [12] took this approach one step further and identified 12 different glycosides of two flavonols present in hops by using parent-MS–MS.

MS was already used to identify catechin metabolites in 1980 [13], but after the introduction of ESI in the mid-1980s [14], ESI-MS and LC–ESI-MS became increasingly popular for the analysis of flavonoids. The recent development of LC–ESI-MS and LC–ESI-MS–MS instruments with high sensitivities, user-friendly software, and relatively low prices has boosted the popularity of these analytical techniques [15]. In this paper we report that LC–ESI-MS–MS can be used to identify and quantify very low levels of theaflavin in biological matrices.

2. Experimental

2.1. Materials

A freeze–dried preparation containing 17.7% (w/w) theaflavin, 31.8% theaflavin-3-gallate, 16.7% theaflavin-3′-gallate and 31.4% theaflavin-3,3′-digallate was a generous gift from Dr. D.A. Balentine, T.J. Lipton (Englewood Cliffs, NJ, USA). (−)-Epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, β-glucuronidase with a high sulfatase activity from *Helix pomatia* (100 000 units ml^{-1} and 7500 units ml^{-1}, respectively), catechol-*O*-methyltransferase (EC 2.1.1.6, from porcine liver), *S*-adenosyl-L-methionine, glutathione, phenolphtalein glucuronide, *p*-nitrophenyl sulfate, and frozen bovine serum were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, for spectroscopy, L-ascorbic acid and Na_2EDTA were from Merck (Darmstadt, Germany). All other materials were obtained from Fisher Scientific (Loughborough, UK) and were analytical grade.

2.2. Healthy volunteers

Two healthy volunteers (one male, one female, aged 40 and 33 years, respectively) refrained from drinking tea for at least 48 h. On the morning of the experiments the volunteers consumed 700 mg mixed theaflavins (approximately 1 mmol) dissolved in 150 ml hot water and supplemented with 20–40 ml lemonade syrup to improve palatability. This amount of theaflavins is equivalent to approximately 30 cups of black tea. Except for not consuming tea for the next 24 h, no further dietary restrictions were imposed.

Blood was collected in EDTA containing vacuum tubes. Blood samples were collected before drinking theaflavins ($t=0$) and 20 min and 1, 2, 4, 7 and 24 h after consumption of theaflavins. Blood was centrifuged at 2500 *g* for 10 min at room temperature. The plasma was collected, and 10% (v/v) of an ascorbic acid solution was added. This solution consisted of 20% (w/v) ascorbic acid, 0.4 mol l^{-1} Na_2HPO_4 and 0.1% (w/v) Na_2EDTA, adjusted to pH 3.6 using 10 mol l^{-1} NaOH. After the addition of the ascorbic acid solution, the pH of the plasma samples was approximately 5.0. Urine samples were collected before drinking theaflavins ($t=0$) and 1, 2, 4, 7 and 24 h after consumption of the theaflavins. All urine samples were adjusted to a pH of 5.0 by addition of the ascorbic acid solution mentioned above. Plasma and urine samples were frozen under nitrogen gas and stored at −20°C. Preliminary

experiments had indicated that spiked theaflavins were not stable in untreated plasma. Only 25% could be recovered after overnight storage at −20°C. After addition of the ascorbic acid solution, however, recoveries of spiked theaflavin increased to 82% (data not shown).

In order to prepare a control plasma containing catechins, the male volunteers consumed 3 g of green tea extract and 2 h later a blood sample was taken and stored as described above.

2.3. Sample treatment

To 1 ml plasma or urine (supplemented with ascorbic acid, pH approximately 5.0) 100 μl β-glucuronidase/sulfatase was added and the mixture was incubated at 37°C for 1 h in closed, nitrogen gas flushed tubes. Preliminary experiments using plasma samples spiked with phenolphtalein glucuronide or *p*-nitrophenyl sulfate indicated an almost complete digestion under the conditions employed. The enzyme preparation was found to have a tannase activity that resulted in the de-gallation of the gallated catechins and theaflavins present in the plasma and urine samples.

After cooling the samples to room temperature, 500 μl acetone was added, samples were vortex-mixed, 2 ml ethyl acetate was added, samples were vortex-mixed again and centrifuged for 5 min at 2500 *g*. The upper ethyl acetate phase was collected. After a second extraction of the water phase with 2 ml ethyl acetate, the organic fractions were pooled and evaporated at 37°C under a stream of nitrogen gas. The residues were dissolved in 50 μl HPLC starting eluent supplemented with 1 mmol l^{-1} ascorbic acid. After centrifugation, 20 μl of the supernatant was injected onto the HPLC system.

Loss of the very low amounts of theaflavins in the samples during storage, deconjugation, and extraction were estimated by spiking theaflavin-free plasma with theaflavin at two levels: 1000 and 50 μg l^{-1}. These samples were stored, deconjugated, extracted and analysed together with the analytical samples. Recoveries were 70 and 50%, respectively.

In order to determine the retention time of the methylated (−)-epigallocatechin, it was prepared essentially as described by Zhu et al. [16].

2.4. HPLC

Standards, plasma extracts and urine extracts were separated on a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany). The Symmetry C_{18} reversed-phase column (150×2.1 mm, particle size 3.5 μm) was from Waters (Milford, MA, USA) and the Spherisorb ODS2 and Inertsil ODS2 columns (250×4.6 mm, 5 μm particle size) were obtained from Chrompack International (Middelburg, The Netherlands). Columns were operated at 30°C.

For LC–scanning-MS and LC–parent-MS–MS analyses, a gradient of mobile phase A (water–acetonitrile–acetic acid, 96:2:2, v/v) to mobile phase B (acetonitrile–acetic acid, 98:2, v/v) was used. Standards and samples were separated by a gradient from 0% B to 74% B over 50 min followed by 100% B for 6 min. For LC–single ion recording-MS (LC–SIR-MS) and LC–multiple reaction monitoring MS–MS (LC–MRM-MS–MS) analyses an isocratic elution of mobile phase A–mobile phase B (77:33) was used. The flow-rate in all experiments was 0.3 ml min^{-1}.

2.5. Mass spectrometry

The Micromass Quattro II (Micromass, Manchester, UK) triple quadrupole MS system was operated using the ESI source. All measurements were carried out using positive ESI, except for LC–SIR-MS, which was done in the negative ESI mode.

For optimisation of the MS parameters an approximately equimolar test mixture of the four (−)-epicatechins and the four theaflavins, dissolved in HPLC mobile phase A, was used. For optimisation of the LC–parent-MS–MS analyses the test mixture was separated on an LC column using the gradient HPLC system described above. Repetitive on-column injections of the test mixture (approximately 70 μmol l^{-1} $compound^{-1}$), were made at varying parameter settings. For the LC–scanning-MS and the LC–parent-MS–MS a source temperature of 120°C and a desolvation temperature of 250°C were optimal. Nitrogen was used as nebulizer gas and drying gas at flow-rates of 400 and 25 l h^{-1}, respectively. The capillary voltage was 4.0 kV and the cone voltage was ramped from 21 to 44 V over a range of

150 to 2000 u. For LC–scanning-MS the scan range was 250 to 2000 u, the scan speed 2 s scan^{-1} and the interscan time 0.03 s. For the LC–parent-MS–MS the scan range of MS1 was 250 to 2000 u, the daughter ion detected in MS2 was at *m/z* 139, the scan speed 2 s scan^{-1} and the interscan time 0.03 s. The collision gas was argon at a pressure of $2.3 \cdot 10^{-3}$ mbar and a collision energy of 27 eV.

For the optimisation of the LC–SIR-MS parameters repetitive on-column injections of the test mixture were made using the isocratic HPLC system described above. A source temperature of 100°C and a desolvation temperature of 300°C were optimal for the LC–SIR-MS analysis. The optimum nitrogen gas flow was 200 l h^{-1} for the drying gas and 30 l h^{-1} for the nebulizer gas. Capillary voltage was 4.0 kV and the cone voltage was 40 V. The dwell time was 0.5 s, the inter-channel delay 0.03 s and the span 0.2 u. For SIR-MS the SIR mass was 562.9 (negative ESI).

For the optimisation of the LC–MRM-MS–MS the source settings obtained in LC–SIR-MS optimisation and the collision energy and collision gas pressure obtained from the LC–parent-MS–MS optimisation were used. The settings for the parent mass and the daughter mass were 565.0 and 139.0 u, respectively.

3. Results

3.1. Choice of HPLC column

Initially, two standard C_{18} HPLC columns (Spherisorb ODS2, Inertsil ODS2) that were frequently used in flavonoid analysis were tested. When low levels of theaflavin were injected, these columns retained this flavonoid infinitely. In contrast, the Symmetry C_{18} column displayed no secondary retention effects with theaflavins, even when very low levels (nmol l^{-1} range) were injected. Therefore, this column was used in all further experiments.

3.2. Continuous flow MS experiments

A test mixture containing equimolar amounts of the four (−)-epicatechins and the four theaflavins was measured in ESI and APCI (atmospheric pressure chemical ionisation) in positive and negative modes. Several post-column additives (sodium iodide, lithium chloride, diammonium citrate, ammonium acetate, acetic acid) were tested during continuous introduction of the test mixture (20 μmol l^{-1} compound^{-1}). The best results were obtained using ESI without post-column addition. Daughter-ion-MS–MS was performed on all eight model compounds. The fragmentation mechanism of (−)-epicatechins and theaflavins as deduced from in the daughter ion analysis in positive-ESI are shown in Figs. 1 and 2, respectively.

With exception of (−)-epicatechin gallate, cleavage a, yielding fragment ion *m/z* 139, gave the highest relative intensity in all catechin and theaflavin daughter-MS–MS spectra (Table 1). In negative ionisation mode, *m/z* 125 was the fragment ion with the highest intensity, but due to the large amount of fragments formed, this ion represented only 12% of the parent ion.

3.3. LC–ESI-parent-MS–MS

Different amounts of theaflavin (0.3–30 mg l^{-1}) were injected on-column (Fig. 3). The detection limit of pure theaflavin, defined as a signal-to-noise ratio of 3 to 1, was 60 μg l^{-1} for the UV signal, and 300

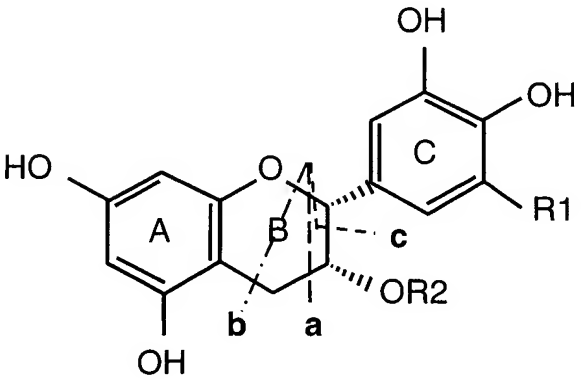

Fig. 1. Proposed fragmentation mechanism of (−)-epicatechins in positive ESI-collision induced dissociation-daughter-ion-MS–MS as adapted from Lin et al. [22]. Analysis was performed on (−)-epicatechin (R1=H, R2=H), (−)-epigallocatechin (R1=OH, R2=H), (−)-epicatechin gallate (R1=H, R2=gallic acid) and (−)-epigallocatechin gallate (R1=OH, R2=gallic acid). Gallic acid=3,4,5-trihydroxybenzoic acid. *m/z* values of the resulting daughter ions of the model compounds are given in Table 1.

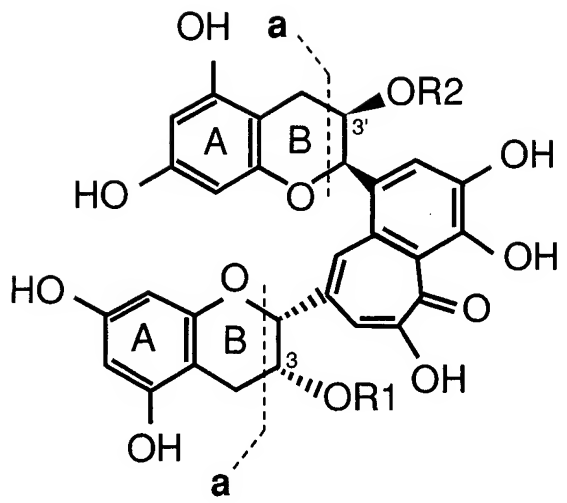

Fig. 2. Proposed fragmentation mechanism of theaflavins in positive ESI-collision induced dissociation-daughter-ion-MS–MS. Analysis was performed on theaflavin (R1=H, R2=H), theaflavin-3-gallate (R1=gallic acid, R2=H), theaflavin-3′-gallate (R1=H, R2=gallic acid) and theaflavin-3,3′-digallate (R1=R2=gallic acid). Gallic acid=3,4,5-trihydroxybenzoic acid. Line a denotes the proposed cleavages during collision induced dissociation. *m/z* values of the resulting daughter ions of the model compounds are given in Table 1.

$\mu g\ l^{-1}$ for the LC–parent-MS–MS signal. In theory, the detection limits for plasma and urine samples were 20-fold lower because the dried residue of the extraction of 1 ml plasma or urine was dissolved in 50 μl before injection. The background signal of the plasma and urine extracts, however, limited the sensitivity severely.

In the extract of a control sample taken after consumption of green tea, peaks of (−)-epicatechin and (−)-epigallocatechin (gallates were removed during enzymatic deconjugation) could easily be detected (Fig. 4). In addition, an ion with a parent *m/z* 321, a daughter of *m/z* 139 and a retention time of 21 min was detected. This ion was identified as methylated (−)-epigallocatechin.

A HPLC peak that produced the characteristic fragment of *m/z* 139 in LC–parent-MS–MS analysis was detected in the urine samples from the male volunteer collected at 1, 2, 4 and 7 h after theaflavin consumption. The $[M+H]^+$ parent ion producing the fragment had a *m/z* of 565. Together with the retention time of 47 min, these values indicated that the parent molecule was unmodified theaflavin. No gallated theaflavins were detected, presumably because the gallate group was removed during the enzymatic deconjugation reaction. The maximum urinary theaflavin concentration was estimated to be 10 $\mu g\ l^{-1}$, which is just below the calculated detection limit of 15 $\mu g\ l^{-1}$. Careful inspection of the traces of the urine samples of the female volunteer indicated that theaflavin might also be present. In the extracts of the plasma samples no theaflavin could be detected.

3.4. LC–SIR-MS

Theaflavin ionises in both positive and negative ESI modes with similar sensitivities. Since negative ESI is more selective, the LC–SIR-MS method was set-up in the negative mode. The dynamic range of the optimised LC–SIR-MS was at least three orders of magnitude (6–6000 $\mu g\ l^{-1}$) and the detection limit of pure theaflavin was 6 $\mu g\ l^{-1}$ on-column.

Table 1
Collision induced-positive ESI-daughter ion-MS–MS of (−)-epicatechins and theaflavins

Compound	Parent ion (*m/z*)	Cleavage a (*m/z*)	Cleavage b (*m/z*)	Cleavage c (*m/z*)	Others (*m/z*)
Epicatechin	291	139 (100)	165 (40)	123 (65)	151 (8)
Epicatechin gallate	443	139 (10)	165 (5)	123 (100)	151 (20), 273 (10), 291 (5)
Epigallocatechin	307	139 (100)	181 (15)	139 (100)	151 (12), 169 (5)
Epigallocatechin gallate	459	139 (100)	181 (3)	139 (100)	151 (38), 289 (20), 307 (5)
Theaflavin	565	139 (100)	–	–	151 (6), 427 (20)
Theaflavin-3-gallate	717	139 (100)	–	–	151 (9), 153 (20) 579 (17)
Theaflavin-3′-gallate	717	139 (100)	–	–	151 (8), 153 (25) 579 (22)
Theaflavin-3,3′-digallate	869	139 (100)	–	–	151 (8), 153 (38), 333 (22)

Intensities (in parentheses) are given as percentages relative to the most abundant fragment.

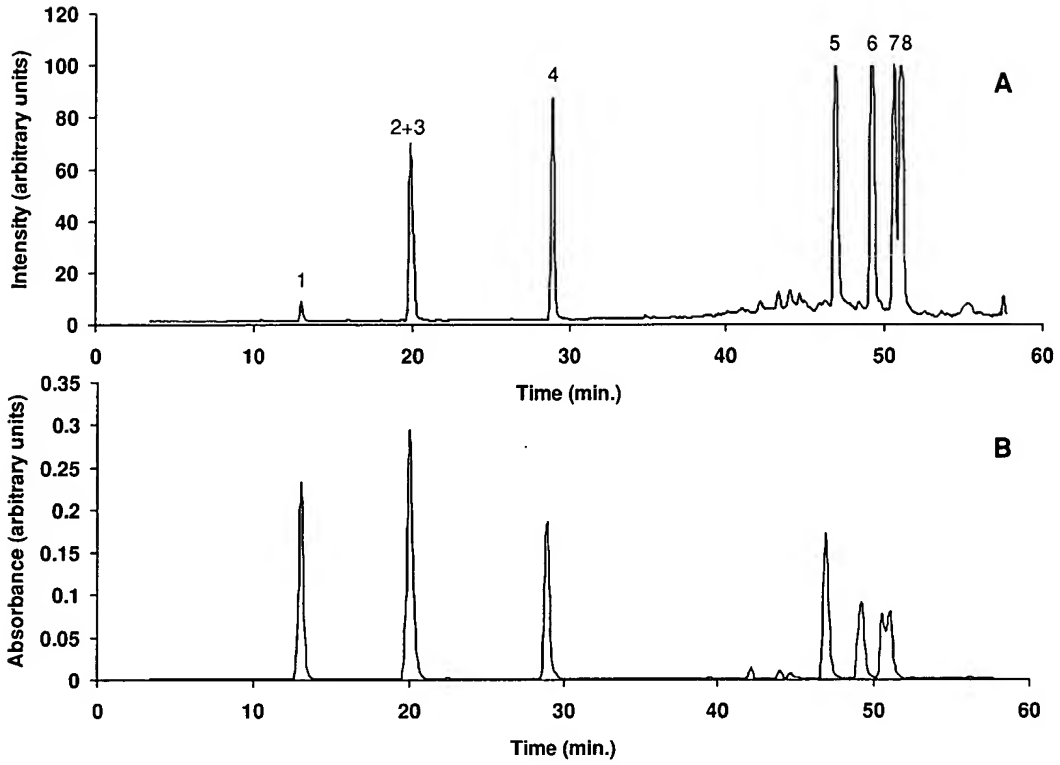

Fig. 3. Injection of an equimolar mixture of black tea flavonoids in a concentration of approximately 70 pmol μl^{-1} compound^{-1} on column. (A) Parents of *m/z* 139 monitored using LC–ESI-parent-MS–MS. (B) UV trace at 280 nm. Peaks in order of elution: (1) (−)-epigallocatechin; (2) (−)-epicatechin co-eluting with (3) (−)-epigallocatechin gallate; (4) (−)-epicatechin gallate; (5). theaflavin; (6) theaflavin-3-gallate; (7) theaflavin-3′-gallate; (8) theaflavin-3,3′-digallate.

However, when the sample extracts were injected, a significant, variable background signal at *m/z* 565 limited the detection of theaflavins severely.

3.5. LC–MRM-MS–MS

Injections of mobile phase containing different concentrations of pure theaflavin (0.6–6000 $\mu g\ l^{-1}$) were made on-column. The standard curve was linear and the dynamic range was at least three orders of magnitude (Pearson correlation coefficient 0.996). The detection limit (signal-to-noise ratio of more than 3:1) of pure theaflavin was approximately 1 $\mu g\ l^{-1}$ injected on-column. Thus, the detection limits could theoretically be as low as 0.05 $\mu g\ l^{-1}$ for plasma and urine samples.

Theaflavin could be detected in all plasma and urine samples collected 1, 2 and 4 h after consumption (Fig. 5). Maximum theaflavin levels detected in the plasma of the female and the male volunteer were 1.0 and 0.5 $\mu g\ l^{-1}$, respectively, and maximum urine levels were 0.6 and 4.2 $\mu g\ l^{-1}$, respectively.

The average relative standard deviation between the duplicates analysed was 4.4 and 17.1% for the plasma samples and the urine samples, respectively.

4. Discussion

There is a rapidly growing number of papers dealing with the application of LC–MS in the analysis of natural substances in food (see Careri et

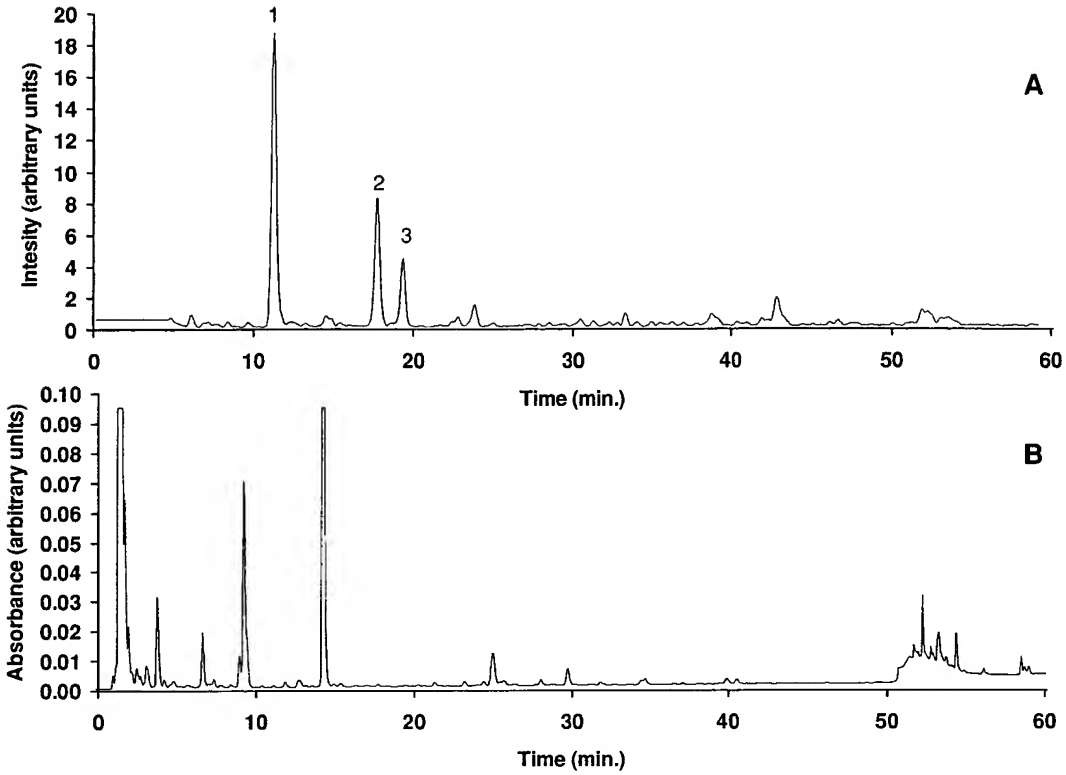

Fig. 4. Analysis of an extract from a deconjugated plasma sample obtained 2 h after the consumption of green tea. The extract separated by HPLC and analysed using UV absorption and parent-MS–MS. (A) Parents of *m/z* 139 as monitored using LC–ESI-parent-MS–MS. (B) UV trace at 280 nm. Peaks in order of elution: (1) (−)-epigallocatechin; (2) (−)-epicatechin; (3) 3-*O*-methyl-(−)-epigallocatechin.

al. [15] for review). Both LC–MS [17–19] and LC–MS–MS [20,21] have been applied for the analysis of catechins in tea extracts. Lin et al. [22] analysed a mixture of four catechins by thermospray LC–MS–MS. The collision-induced dissociation spectra they reported are almost identical to those obtained in the present study. A very similar fragmentation behaviour of catechins during electron-ionisation MS was reported recently by Miketova et al. [23]. The fragmentation behaviour of theaflavins, as reported by Chen et al. [24] using LC–APCI-MS, was also very similar to the pattern we proposed.

Apart from these mechanistic MS studies, LC–MS can also be used to detect the low concentrations of catechins [25] and quercetin-glycosides [26] in plasma, or isoflavones in urine [27]. These methods had detection limits of 100 mg l^{-1}, 300 μg l^{-1} and 5 μg l^{-1}, respectively. Coward et al. [28] developed an LC–MRM-MS–MS method for the measurement of isoflavones in plasma with a detection level of 0.3 μg l^{-1}. The detection limit of our LC–MRM-MS–MS was approximately 0.05 μg l^{-1} theaflavin. This high sensitivity can, at least partly, be explained by the lower background signal obtained by the use of LC–MRM-MS–MS instead of LC–SIR-MS.

The maximum theaflavin concentration detected in plasma was 1.0 μg l^{-1}. This level must be at least doubled to correct for the low recovery at these minute levels of theaflavin. Corresponding values for catechins in plasma are more than a 100-fold higher. Lee et al. [7] reported maximum plasma catechin levels of 500 μg l^{-1} after consumption of 235 mg catechins. Yang et al. [8] reported maximum plasma catechin concentrations of 300 μg l^{-1} after an oral dose of 300 mg catechins. Plasma half-life of theaflavin seemed to be in the order of 1–3 h, which

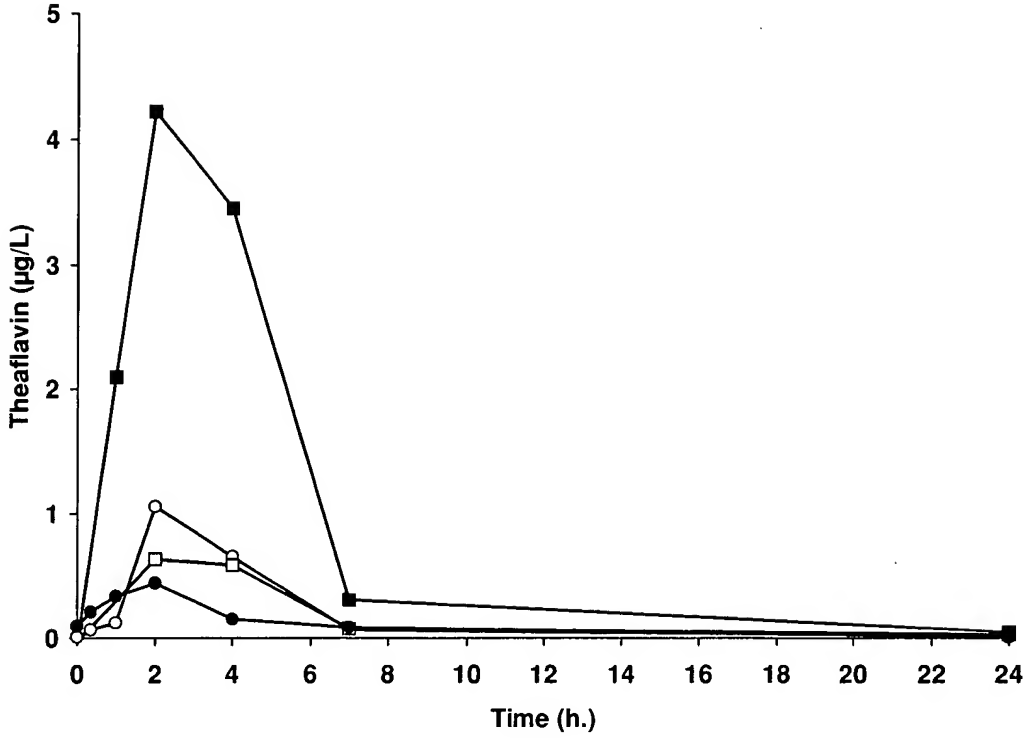

Fig. 5. Theaflavin concentrations in plasma and urine samples from two healthy volunteers who consumed 700 mg mixed theaflavins. Samples were treated with glucuronidase/sulfatase and extracted into ethyl acetate. The dried ethyl acetate extracts were re-dissolved and separated using reversed-phase HPLC. Theaflavin was detected using LC–MRM-MS–MS at parent ion *m/z* 565, daughter ion *m/z* 139. Open symbols=female volunteer, closed symbols=male volunteer. Circles=plasma, squares=urine.

is similar or slightly shorter than the values reported for catechins [8].

The maximum urinary theaflavin concentration was 4.2 µg l^{-1}. Total urinary theaflavin excretion during 24 h was calculated to amount to 4 µg theaflavin or less. This represents 0.0006% or less of the dose administered. In the study by Lee et al. [7] approximately 5 mg of the 235 mg (or 2%) of catechins ingested was recovered in 24 h urine.

A major drawback of the current study is that the assay can only detect theaflavin metabolites with an unmodified A and C ring. Metabolites, in which these ring systems were modified, escaped detection. Only uniformly carbon-labelled flavonoids will allow detection of nearly all substances that are derived from the labelled precursor. Until these labelled theaflavins are available the conclusion must be that consumption of black tea does not lead to significant levels of intact theaflavins in the blood.

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Stability of tea theaflavins and catechins

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Abstract

Green tea catechins (GTC) and theaflavins (TF) possess a variety of biological activities. The present study focused on stability of GTC and TF in various solutions and drinks. It was observed that both GTC and TF were vulnerable to degradation caused by elevation of temperature and pH of incubation media. In general, GTC was more stable than TF. When boiled in water, four GTCs showed similar rates of degradation, but in sodium phosphate buffer (pH7.4) at room temperature, four GTCs demonstrated varying stability, with epigallocatechin gallate (EGCG) and epigallocatechin (EGC) being completely degraded in 6 h of incubation, while epicatechin (EC) and epicatechin gallate (ECG) were only degraded by less than 35%. Four TFs also demonstrated varying stability, with theaflavin-3,3′-digallate (TF3) and theaflavin-3′-gallate- B (TF2B) in general being more stable than theaflavin-1 (TF1) and theaflavin-3-gallate-A (TF2A) in either boiling water or alkaline sodium phosphate buffer. When incubated in various solutions and soft drinks, both GTC and TF had poor long-term stability and decayed by at least 50% during the first month of storage at room temperature.

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Keywords: Catechins; Theaflavins; Stability; Tea drinks

1. Introduction

Green tea catechins (GTC) are the principal components in tea leaves and comprise mainly four compounds namely (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG). During the production of black tea, extensive enzymatic oxidation of the leaf catechins leads to brown products such as theaflavins (TF) and thearubigins (TR). The major TF in black tea are theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3′-gallate (TF2B), and theaflavin-3,3′-digallate (TF3). Both GTC and TF have been subjects of extensive research for their anticarcinogenic, hypolipidemic and antioxidant activities (Ahmad & Mukhtar 1999; Chan, Fong, Huang, Ho, & Chen, 1999; Leung, Su, Chen, Zhang, Huang, & Chen, 2001).

Stability of GTC and TF in tea beverage has not received much attention. Although GTC and TF have many beneficial effects, their decomposition must be prevented when tea drinks are produced, stored and transported. Our previous study demonstrated that stability of GTC, as a whole, is pH-dependent; in alkaline solution, it is very unstable and decomposes in a few minutes, whereas, in acidic solution, it is relatively stable (Zhu, Zhang, Tsang, Huang, & Chen, 1997). It was also observed that the four catechins showed varying stability with EGCG and EGC being more unstable than EC and ECG, irrespective of pH. Another study also analysed the composition of bottled and canned tea drinks available in the market and found that the four catechins were present in low quantities, having been mainly converted to their corresponding epimers (Chen, Zhu, Tsang, & Huang, 2001). However, there is no study to date that has examined the stability of theaflavins in black tea. The objective of the present study was to study the degradation of TF compared with that of GTC under various conditions.

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2. Materials and methods

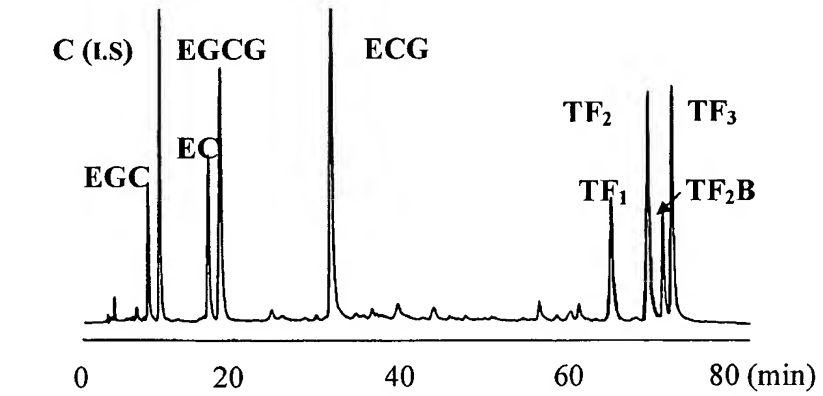
2.1. Preparation of GTC–TF mixture

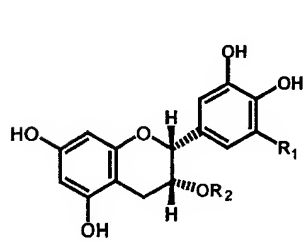
TF extract was prepared with Keemun black tea (Shanghai Tea Import & Export Corporation, China), as previously described (Leung et al., 2001). In brief, the tea leaves (2.25 kg) were firstly extracted three times using 13.5 l of 70% ethanol. The ethanol was then removed in a rotary evaporator. The remaining aqueous solution was extracted subsequently using chloroform (3 l), ethyl acetate (2 l), and butanol (2 l). The ethyl acetate extract was applied onto a silica gel column (30×2 cm i.d.; silica gel 60 M, 230–240 mesh). Total TF fraction was obtained when the column was eluted with 4 litres of chloroform-ethyl acetate (1:1, v/v), followed by 4 l of chloroform-ethyl acetate (4:1,v/v), as previously described (Leung et al., 2001)

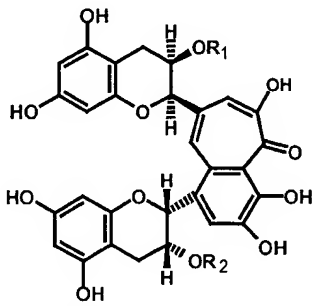
GTC extract was prepared with Longjing green tea (Huangshan Forestry Farm, Xiaoshan, Zhejiang, China), as previously described (Agarwal, Katiyar, Zaidi, & Mukbtar, 1992). In brief, 10 g of dry longjing tea leaves were extracted three times with 140 ml of hot distilled water (80 °C). The infusion was then cooled to room temperature, filtered and extracted with an equal volume of chloroform to remove caffeine and pigments. The remaining aqueous layer was then extracted twice with an equal volume of ethyl acetate. The total GTC extract was obtained after the removal of ethyl acetate in a rotary evaporator. The TF–GTC mixture was prepared by mixing equal amounts of GTC and TF extracts.

2.2. HPLC analysis of catechins and theaflavins

The individual catechins and theaflavins were quantified using a Shimadzu LC-10AD HPLC (Tokyo, Japan), equipped with a ternary pump delivery system, as described previously. In brief, the mixture (10 μl, 0.5 mg/ml) was injected onto column (Hypersil ODS, 250×4.6 mm, 5 m, Alltech, Deerfield, IL, USA) via a Rheodyne valve (20 μl capacity, Shimadzu, Tokyo, Japan). The mobile phase consisted of 2% acetic acid in water (v/v) (Solvent A) and acetonitrile (Solvent B). After the injection of the sample, Solvent B was increased from 8 to 15% over 28 min, to 31% over an additional 52 min and then back to the starting ratio over an additional 5 min. The flow rate was maintained at 1.0 ml/min. The individual catechin and TF were monitored at 280 nm and quantified using (+)-catechin as an internal standard (Fig. 1).







EGC = Epigallocatechin R_1=OH R_2=H
EC = Epicatechin R_1=H R_2=H
EGCG = Epigallocatechin gallate R_1=OH R_2=galloyl
ECG = Epicatechin gallate R_1=H R_2=galloyl

TF_1 = Theaflavin-1 R_1=R_2=H
TF_2A = Theaflavin-3 -gallate-A R_1=galloyl R_2=H
TF_2B = Theaflavin-3'-gallate-B R_1=H R_2=galloyl
TF_3 = Theaflavin-3,3'-digallate R_1=R_2=galloyl

Fig. 1. Structures and HPLC eluting pattern of green tea cateachins and theaflavins.

2.3. Thermal stability of GTC and TF

The GTC–TF mixture (5 mg) was dissolved in 10 ml of doubly deionised water and delivered into each Pyrex tube (10×1.6 cm, i.d.). The stabilities of individual GTC and TF were examined at 24, 70, and 100 °C for 3 h in open air without any agitation. During the incubation, 0.5 ml were periodically sampled and mixed with 0.2 ml (+)-catechin solution (0.5 mg/ml) as an internal standard. The mixture was extracted with 1 ml of ethyl acetate. After the removal of ethyl acetate under a gentle stream of nitrogen, 0.5 ml of distilled water were added to dissolve the GTC and TF. The sample was then subjected to HPLC analysis, as previously described.

2.4. pH stability of GTC and TF

The pH stability of GTC and TF was assessed by incubating 5 mg GTC-TF mixture in 10 ml of 60 mM sodium phosphate buffer at different pH values, varying from 5.0 to 7.4, in a Pyrex test tube (10×1.6 cm, i.d.) in open air without any agitation. An aliquot (0.5 ml) of the incubation mixture was periodically sampled and 0.2 ml of (+)-catechin solution (0.5 mg/ml) were added, followed by extracting twice with 1 ml ethyl acetate. After evaporation, the GTC and TF were dissolved into 0.5 ml water and subjected to HPLC analysis.

2.5. Long-term stability of GTC and TF

To study the long-term stability of GTC and TF in tea drinks, The GTC–TF mixture (0.5 mg/ml) was incubated in sodium phosphate buffer solutions with varying pH (4–5), a sucrose solution (0.15 g/ml), a sucrose-citric acid solution (0.15 g sucrose/ml, 2 mg citric acid/ml), and four kinds of commercial soft drinks, namely, Coca cola, 7 UP, Pepsi and Sprite. Each solution containing GTC–TF mixture was autoclaved at 120 °C for 20 min, sealed in a sterilized Pyrex tube (10×1.6 cm, i.d.) and placed in the dark at room temperature for six months. An aliquot (4 ml) of the solution was sampled monthly and mixed with 1 ml of 0.4 mg/ml (+)-catechin solution as an internal standard. The sample was extracted twice with 4 ml of ethyl acetate. After the removal of ethyl acetate under a gentle stream of nitrogen, the GTC-TF was dissolved in 0.5 ml of distilled water and subjected to HPLC analysis, as described previously.

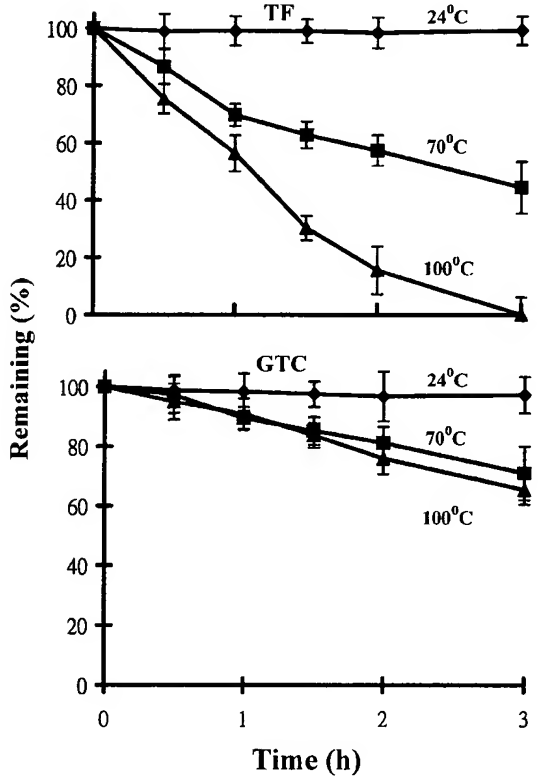


Fig. 2. Thermal stability of green tea catechins (GTC) and theaflavins (TF) in water solutions at 24, 70 and 100 °C. GTC is the sum of EGCG, EGC, ECG and EC. TF is the sum of TF1, TF2A, TF2B and TF3. Data are expressed as means ±S.D. of $n=3$ samples.

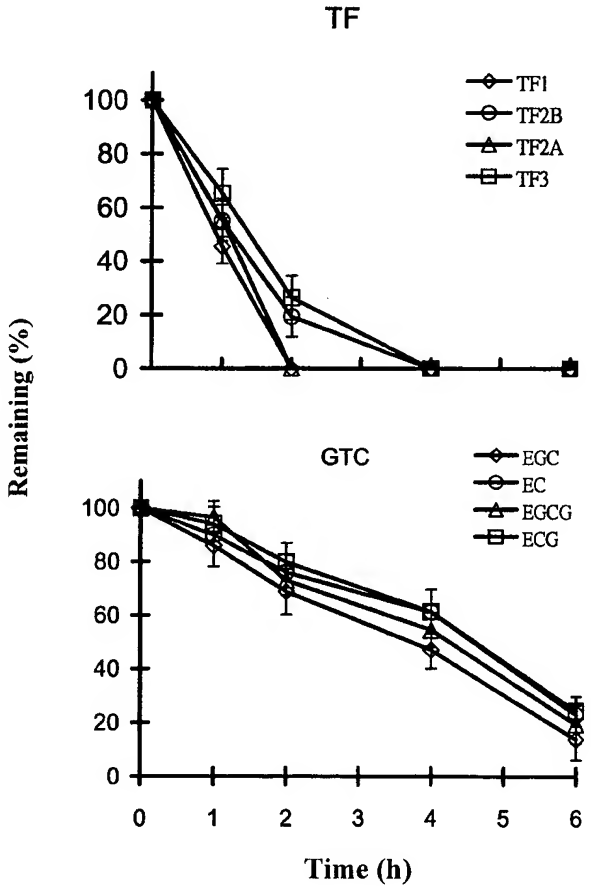


Fig. 3. Thermal stability of individual green tea catechins (GTC) and theaflavins (TF) in boiling water. Data are expressed as means ±S.D. of $n=3$ samples.

3. Results and discussion

3.1. Thermal stability of GTC and TF

GTC and TF demonstrated different thermal stabilities. As shown in Fig. 2, GTC as a sum of EGCG, EGC, ECG and EC was more stable than TF, which is a sum of TF1, TF2A, TF2B and TF3. Heating at 100 °C for 3 h led to 25% degradation of GTC. In contrast, TF was completely degraded (Fig. 2). Heating at 70 °C for 3 h degraded TF 56%, whereas it only destroyed 29% of GTC. It is concluded that GTC in green tea, as a whole, is more stable than TF in black tea.

When individual TFs were compared with those of GTCs, the former were more susceptible to thermal destruction than the latter (Fig. 3). The four GTC derivatives appeared to have similar rates of degradation at 100 °C. Among the four TF derivatives, TF3 and TF2B had relatively slower rates of destruction than the other two (Fig. 3).

Traditional preparations of tea drinks in a porcelain teapot do not degrade very much of the GTCs. In China, tea beverage is simply brewed by pouring 300–400 ml of boiling water onto 4–5 g of dry tea leaves in a tea cup. After approximately 5 min, the tea leaves are saturated and sink to the bottom; the infusion is then ready for serving. As shown in Fig. 2, heating at 100 °C for 30 min degraded only about 5% of the GTC. This observation is similar to that previously reported by Chen, Zhu, Tsang, & Huang (2001), namely, that about 10% of the GTC was lost after 10 min in boiling water. However, destruction of TF reached 25% under the same conditions.

3.2. pH-Dependent stability of GTC and TF

The effect of pH on the stability of GTC and TF was significant at room temperature. As illustrated in Fig. 4, both GTC and TF demonstrated pH-dependent stability; the lower the pH value of the sodium phosphate buffer, the greater the stability. When incubated in buffer of the same pH, TF was much more unstable than GTC. For example, incubation in sodium phosphate buffer of pH 7.4 for 6 h led to 95% destruction of TFs but only to 65% GTC degradation. We have measured the pH values of several bottled and canned tea drinks available on the Hong Kong market. It was found that the pH value of tea drinks varied with brands, ranging from 3.3 to 6.5. As shown in Fig. 4, GTC and TF had

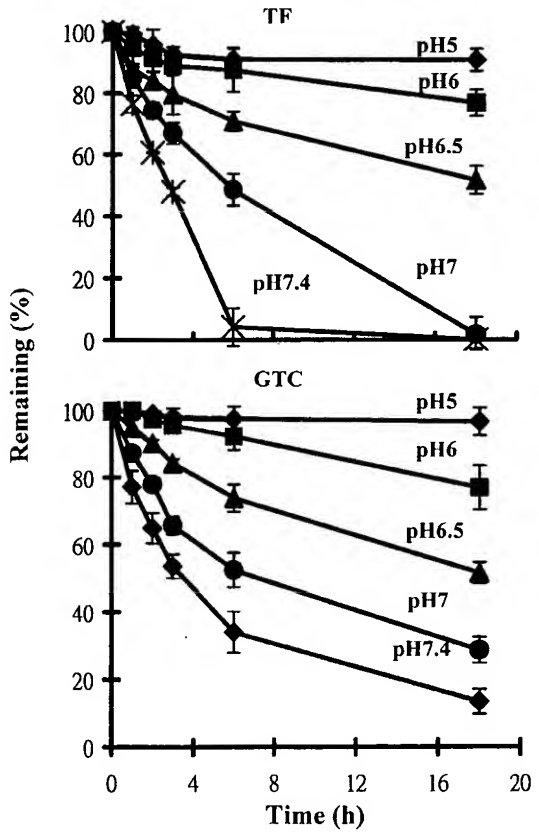


Fig. 4. Stability of green tea catechins (GTC) and theaflavins (TF) in sodium phosphate buffer of various pH. Data are expressed as means ± S.D. of $n = 3$ samples.

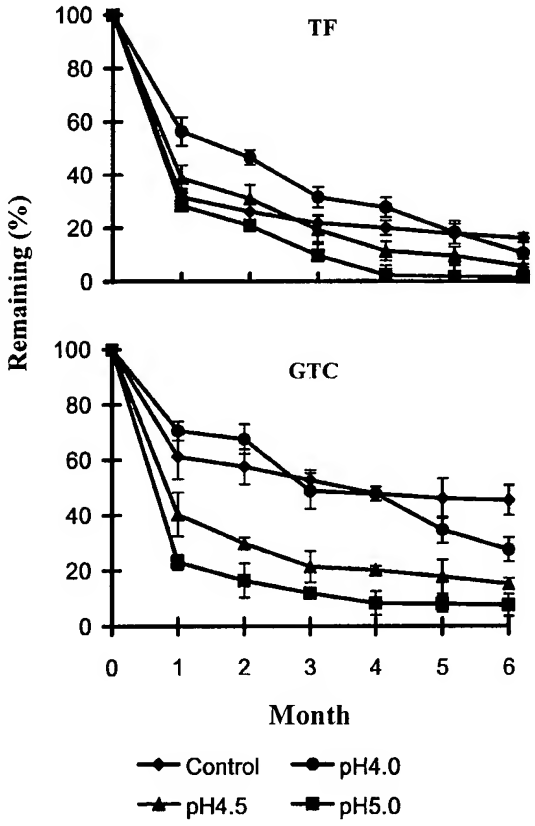


Fig. 5. Long-term stability of green tea catachins (GTC) and theaflavins (TF) in distilled water (control) and sodium phosphate buffer solution with pH 4.0–5.0. Data are expressed as means ± S.D. of $n = 3$ samples.

short-term stability in the tea drinks with pH 5 or less. However, the long-term stability of GTC and TF was not promising. As shown in Fig. 5, 45–73% of TF was lost when incubated in sodium phosphate buffers with pH 4–5 for 1 month. Similarly, incubation for one month led to 30–78% of GTC destruction. The observed pH-dependent stability of GTC was in agreement with the reports by Chen, Zhu, Wong, Zhang, and Huang (1998), Chen, Zhu, Tsang, and Huang (2001), Zhu, Zhang, Tsang, Huang, and Chen (1997), Suematsu, Hisanobu, Saigo, Matsuda, Hara, & Komatsu (1992), and Komatsu (1991). The present study is the first to report that TF also exhibit a pH-dependent stability. The results suggest that the pH-dependent stability has to be taken into consideration when commercial canned green or black tea drinks are produced.

3.3. pH-stability of individual catechin and theaflavin

Individual TFs and GTCs demonstrated differences in stability (Fig. 6). To simplify the presentation, only data for pH 7.4 have been shown. Among the four TFs, TF2B appeared to be more stable for the first three hours of incubation, while TF3 was more resistant to degradation from 3 to 6 h in sodium phosphate buffer (pH 7.4). Among the four GTCs, EGCG and EGC were most unstable, while EC and ECG were relatively stable. EGC was almost completely degraded when incubated for 3 h at pH 7.4, while EGCG was completely destroyed at the end of the 6 h incubation. Under the same incubation conditions, ECG and EC were decreased by 20 and 5%, respectively, after 3 h. The reason that EGCG and EGC were more unstable than ECG and EC is almost certainly due to the three vicinal hydroxyl groups at positions 3′, 4′ and 5′ in EGCG and EGC being more vulnerable to destruction (producing semiquinone free radicals) than the two vicinal hydroxyl groups at positions 3′ and 4′ in ECG and EC (Yoshioka, Sugiura, Kawakara, Fugita, Makino, Kamiya, & Tsuyumu 1991). It was noteworthy that the degradation pattern of the four GTCs in boiling water was different from that in sodium phosphate buffer (pH 7.4) (Fig. 3). In the boiling water, they had similar rates of degradation while, in sodium phosphate buffer (pH 7.4), EGCG and EGC were destroyed much faster than

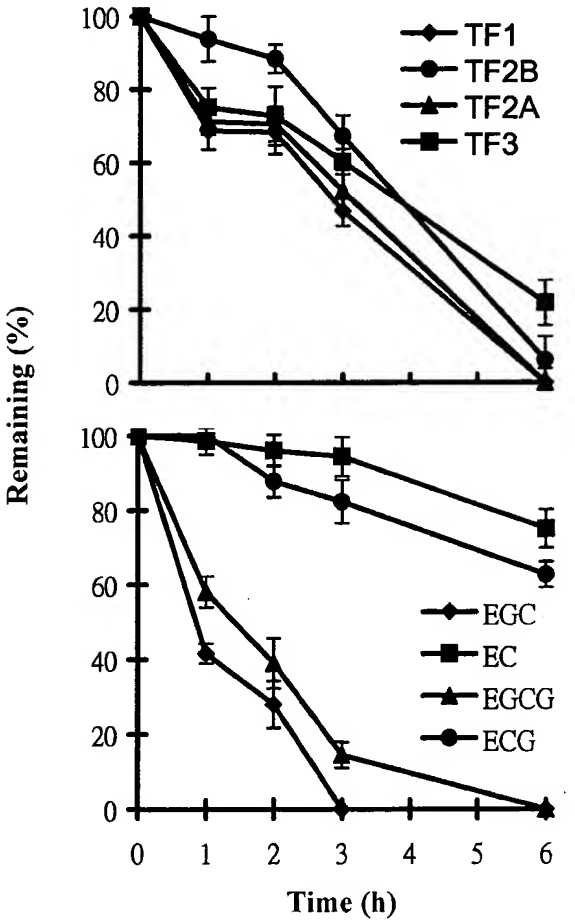


Fig. 6. Stability of individual green tea catechins (GTC) and theaflavins (TF) in sodium phosphate buffer (pH 7.4). Data are expressed as means ±S.D. of $n=3$ samples.

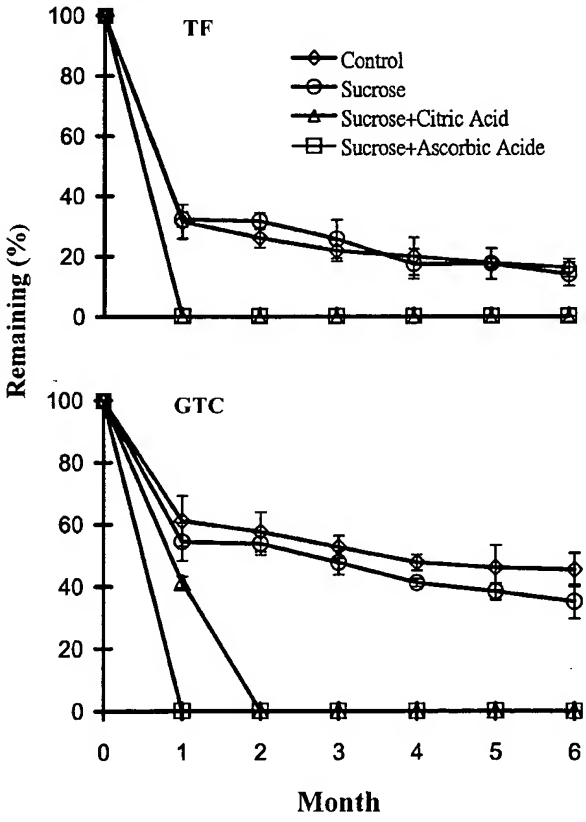


Fig. 7. Long-term stability of green tea catachins (GTC) and theaflavins (TF) in distilled water (control), sucrose solution (0.15 mg/ml), sucrose solution containing citric acid (2 mg/ml) and sucrose solution containing ascorbic acid (11 mg/ml). Data are expressed as means ±S.D. of $n=3$ samples.

ECG and EC. It would be of interest to study the decomposition pathways of the two types of GTC to better understand the behaviour of these active tea ingredients in various in vitro and in vivo media.

3.4. Stability of GTC and TF in drinks

A 6-month assessment of stability of GTC and TF was conducted in various solutions containing sucrose, citric acid and ascorbic acid, that are commonly added to the tea drinks. Compared with the control water solution, addition of sucrose had no or little effect on stability of either GTC or TF (Fig. 7). Addition of citric acid and ascorbic acid destabilised GTC and TF. When the GTC–TF mixture was added to the four soft drinks, they showed a faster degradation than those in the control water solution (Fig. 8). Regardless of the types of solutions and soft drinks, TF was less stable than GTC (Figs. 7 and 8).

There is limited information on long-term stability of GTC and TF in canned and bottled tea drinks. To assure the quality and shelf-life of tea drinks, it is a must to assess the long-term stability of GTC and TF. In addition to being pH-dependent, GTC and TF had poor stability in drinks. The results clearly demonstrate that GTC and TF were degraded by at least 50% within the first month of storage, in either water–sucrose solution or other soft drinks. This may partially explain the low content of GTC in the commercial tea drinks (Chen, Zhu, Tsang, & Huang, 2001). There has been no report on decomposition of GTC and TF except that Zhu, Huang, Yu, LaVoie, Yang, & Ho (2000) isolated and identified three oxidation products when EGCG and EGC interacted with H_2O_2. No study has addressed the possible fates of ECG, EC and TF during heat treatment, except for our previous report (Chen, Zhu, Tsang, & Huang, 2001) which demonstrated that half of the GTC was converted to the corresponding epimers if GTC was heated at 120 °C for 20 min. We are currently studying the decomposition pathway of each GTC and TF and examining whether these decomposition products have any biological functions such as those of their precursors.

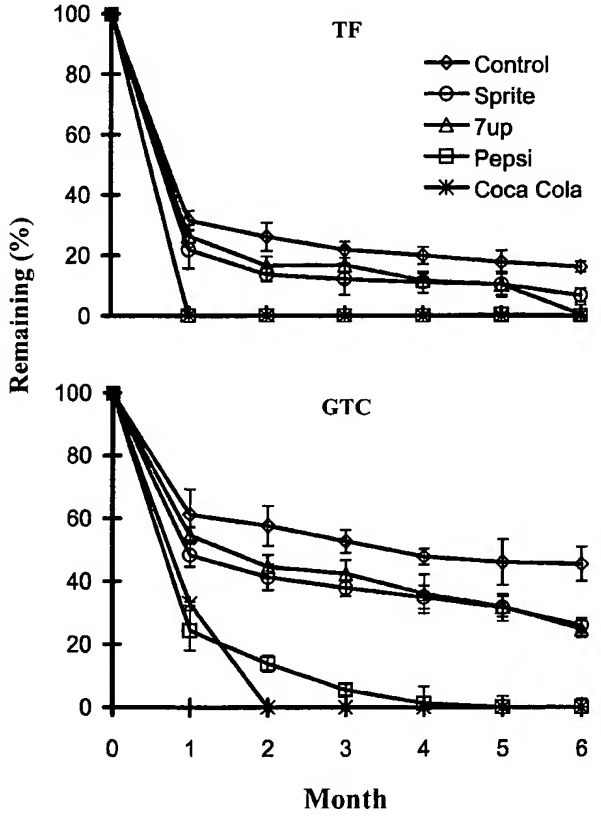


Fig. 8. Long-term stability of green tea catachins (GTC) and theaflavins (TF) in distilled water (control), and soft drinks. Data are expressed as means ±S.D. of $n=3$ samples.

4. Conclusion

Four catechins in green tea and four theaflavins in black tea are believed to be the active ingredients that possess a range of beneficial effects. The present study examined the stability of GTC and TF. In general, TF as a whole is more susceptible to degradation than GTC in boiling water and alkaline solutions. The four GTCs showed varying stability in alkaline pH solution with EGCG and EGC being unstable and ECG and EC being relatively stable. In boiling water, the four GTCs showed similar patterns of destruction. Regarding the four TFs, TF3 and TF2B appeared to be relatively more stable than TF1 and TF2A in both boiling water and alkaline solutions. When GTC and TF were added to various drinks, they were degraded by at least 50% within the first month of storage at room temperature. The observed behaviour of GTC and TF derivatives should be taken into consideration when bottled and canned tea drinks are produced.

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